ENZYME IMMUNOASSAY AND RELATED BIOANALYTICAL METHODS

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INTRODUCTION

Enzyme immunoassay, a bioanalytical method incorporating an antigen–antibody reaction to capture the analyte of interest and an enzyme reporter system to detect the captured analyte, is one of the most widely used immunoassay formats. The method is sometimes applied only qualitatively to indicate the presence of an antigen in a matrix. However, in the more common quantitative implementation, a calibration (standard) curve is incorporated, from which the concentration of the analyte in unknown samples is interpolated. In the decades since the development of a radioimmunoassay for insulin by Yalow and Berson (1), immunoassays have been widely applied in support of medical practice and drug development. However, in recent years, there has been a decline in the application of immunoassays to the quantitation of low-molecular-weight xenobiotics, primarily due to the advent of liquid chromatography–mass spectrometry (LC–MS) methods, which have high sensitivity and specificity. This is particularly so for support of early drug discovery, where assay development times of as little as a day and analytical run times of only a few minutes per sample make LC–MS ideally suited to fast delivery of results to discovery scientists. Nonetheless, the remarkable specificity of antibodies allows their application in well-characterized immunoassays to the support of Phase III and Phase IV clinical trials as a cost-effective alternative to LC–MS methods. In addition, these methods are still widely used for therapeutic drug monitoring and analysis of low-molecular-weight hormones, such as steroids, in support of medical diagnostics. Immunoassays remain the method of choice for the quantitation of protein macromolecules and antibodies in complex matrices. Another major application of immunoassays is in the detection and quantitation of biomarkers, which are evolving to be of pivotal importance in the evaluation of pharmacological, toxicological, and clinical activities of candidate drugs (2).

Immunoassays generally vary in the type of critical antibody binding reagent or the detection and reporter systems used to monitor the end-point of the binding reaction. These different types of immunoassays have many characteristics in common; therefore, this chapter will include discussions of both enzyme immunoassays and other closely related methods. The enzyme immunoassay technique has been the subject of several textbooks, monographs, and review articles, including an excellent, comprehensive discussion in an earlier edition of this series (3). Thus, this chapter does not provide an in-depth review of the mechanistic details for producing and processing antibodies as reagents or on assay conditions for enzyme immunoassay. Rather, the intent is to present this technique in the context of several primary topics, namely the range of bioanalytical applications, the different, and sometimes additional, validation considerations imposed upon an enzyme immunoassay and its fraternal immunoassay methods, and some newer techniques that are complementary to enzyme immunoassay and offer potential performance enhancements. The chapter is written from the perspective of bioanalysis in biological fluids and does not address in any detail other applications of enzyme immunoassay, such as support of process control or product release, although such topics have been addressed elsewhere (4).

FORMAT OF ENZYME IMMUNOASSAYS

The format of an enzyme immunoassay refers to the configuration in which the components of the assay are assembled for routine application. Once this format has been established and assay conditions defined during assay development, they must remain unchanged through validation and subsequent application to sample analysis. Enzyme immunoassay formats fall broadly into two categories, namely heterogeneous and homogeneous. In a heterogeneous assay, at least one key reagent is

immobilized on a solid surface and there is at least one "washing" step before the final detection step. In contrast, in a homogeneous assay, all reagents are in solution together and there is no "washing" step prior to signal generation and detection. Both categories of assay include formats described as competitive and noncompetitive. In a competitive assay, there is direct competition between the labeled and the unlabeled antigen (analyte or ligand) in solution or, in some cases, between immobilized and soluble antigen for a limited number of antibody binding sites. In noncompetitive assays, antibody binding sites to capture and detect the antigen are not limiting because the antigen is incubated with excess capture antibody and enzyme-labeled detection antibody. An example of a competitive homogeneous assay format is the enzyme-multiplied immunoassay (EMIT) system (5), in which enzyme-labeled antigen competes directly in solution with unlabeled antigen in the biological sample (or calibration standard and quality control samples) for a limited number of antibody binding sites. The reaction endpoint is detected and quantitated spectrophotometrically without any intervening wash steps. This assay configuration is shown in Fig. 1. Enzyme-linked immunosorbent assay (ELISA) is an example of a heterogenous noncompetitive immunoassay. In this format, the primary antibody against the analyte of interest is immobilized on a solid plastic surface, usually a multiwell (or microtiter) plate. The biological sample is dispensed into the multiwell plate and incubated. The immobilized antibody then captures the analyte of interest, and the excess analyte is removed by washing. The antigen–antibody complex is then detected by two-step incubation with conjugated antibody and its substrate. First, an enzyme-labeled antibody, directed against the captured analyte, sandwiches the immobilized antibody–antigen complex. In a second incubation with an appropriate enzyme-specific substrate solution, a colored (or fluorescent or chemiluminescent) product is generated and quantitated spectrophotometrically. This assay format is depicted in Fig. 2. The ELISA can also be established in a competitive heterogenous format in which the antigen is immobilized on a multiwell plate and competition is established between the immobilized antigen and the antigen in solution for a limited number of binding sites on the primary antibody, also in solution. Following a fixed incubation period, the plates are washed and incubated with excess enzyme-labeled secondary antibody (directed against immunoglobulin from the species in which the primary, anti-antigen antibody was generated). The endpoint of this competition is then detected, following appropriate washing steps, by incubations with a signal-generating substrate. This assay format is depicted in Fig. 3.

It should be noted that the relationship between the final signal output and concentration of the analyte (dose–response) may be one of direct or inverse proportionality, and is dependent on the specific assay format. In addition, a number of different reporter enzymes may be used (e.g., horseradish peroxidase, alkaline phosphatase, β-galactosidase), along with a number of different signaling systems (e.g., substrates that yield chromogenic or fluorescent or chemiluminescent products, activation of signaling enzymes, amplification by biotin–avidin system or polymerase chain reaction).

ENZYME IMMUNOASSAYS FOR LOW-MOLECULAR-WEIGHT ANALYTES

Although the advent of sensitive LC–MS assays with short development times has reduced the need for immunoassays for low-molecular-weight compounds, the sensitivity, high-throughput and relatively low-cost characteristics of these assays still allow them to play an important role in some cases. Immunoassay support at the drug discovery stage may still be viable in such areas as the evaluation of biomarkers or determination of peptides, in which the elimination of sample cleanup prior to assay may constitute a valuable advantage of immunoassay over LC–MS. In addition, for low-molecular-weight therapeutic candidates, immunoassays can be used to support late-stage (Phases III and IV) clinical trials, when the metabolic pathways and the use of concomitant medications have been clearly defined. Critical to the application of immunoassay for analysis of low-molecular-weight compounds is the development of an antibody with clearly defined specificity for the analyte of interest. Low-molecular-weight compounds (less than 1000–2000 Da) are generally nonimmunogenic, or only weakly immunogenic, when administered directly to animals. To elicit an immune response producing antibodies suitable for use as reagents in immunoassays, these compounds must be conjugated to a carrier protein, e.g., bovine serum albumin (BSA), prior to immunization. This subject has been reviewed in depth previously (3). The immune system of the immunized animal responds to the carrier, and then secondarily to the hapten (the analog of the analyte bound to the carrier protein) attached to it. Although antibodies to the carrier protein are often produced in large amounts, they are easily removed by such techniques as affinity chromatography (6). Conversely, the desired antibodies to the analyte may be removed by affinity chromatography, which may also improve their specificity by eliminating or reducing

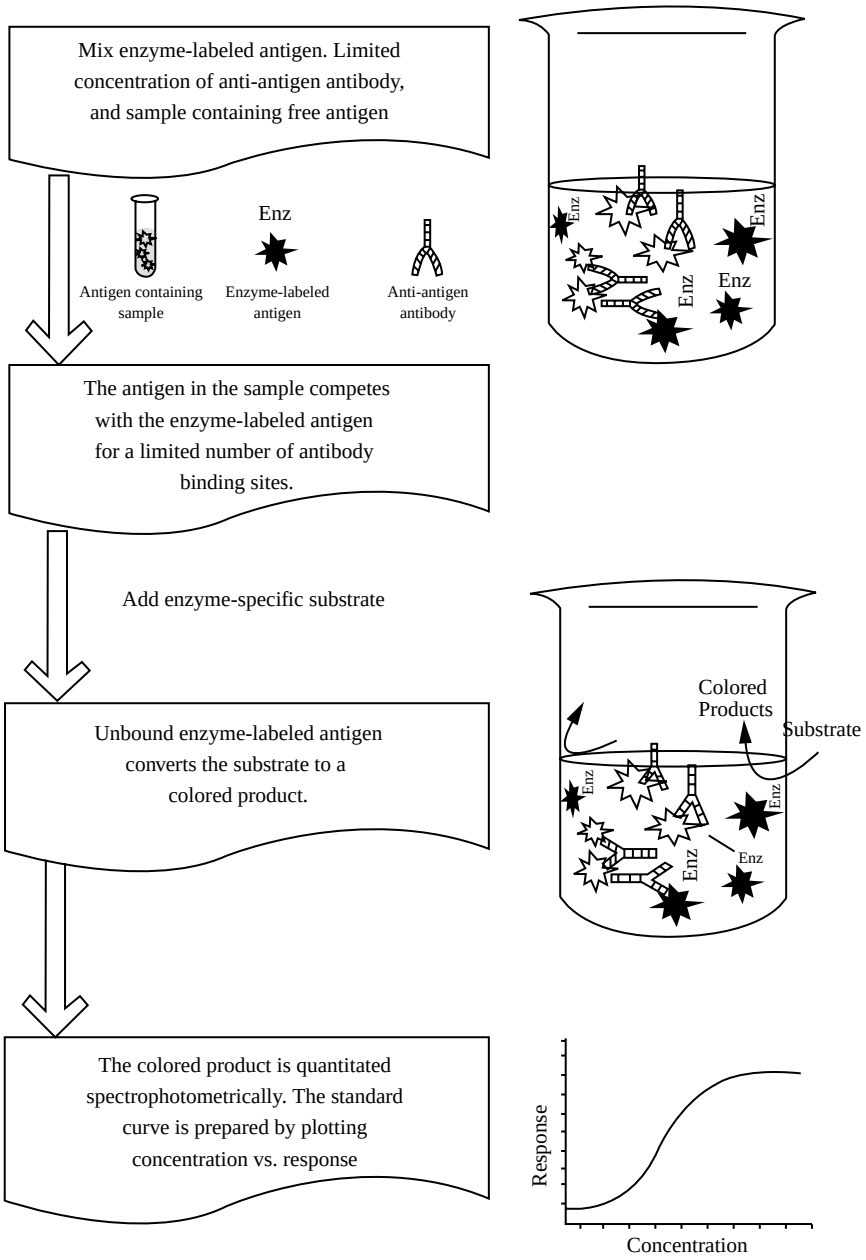

Fig. 1 An illustration of enzyme-multiplied immunoassay.

antibody populations that may cross-react with closely related chemicals, such as metabolites or degradation products. However, in many cases, carrier antibodies do not interfere in the assay to quantitate the analyte of interest and, therefore, anti-antigen antibodies in crude antiserum may be used in immunoassays without further purification. The site of attachment of the hapten to the carrier protein (either directly with the molecule via functional groups that are suitable for chemical coupling, or via a synthetic analog prepared to incorporate chemical coupling functionality) will determine the specificity profile of the resulting antiserum. As a result, the site of attachment of hapten to carrier protein must be selected judiciously, considering all available knowledge of the metabolism of the compound in the animal system under investigation. Metabolic changes closest to the site of attachment will be poorly discriminated from the parent molecule, whereas metabolic changes distant from the conjugation site will be distinguished most clearly (i.e., will have lowest cross-reactivity). The ultimate

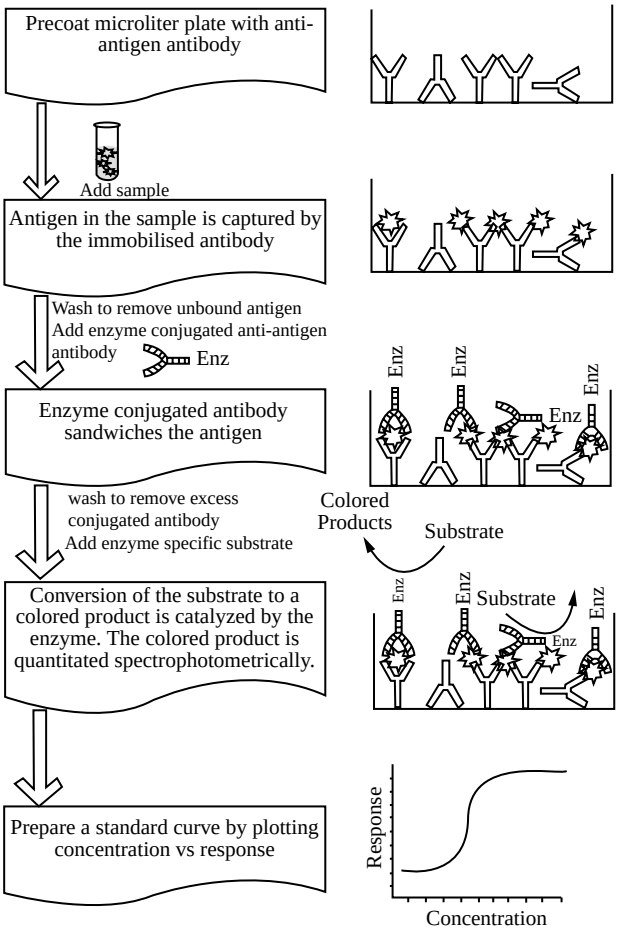

Fig. 2 An illustration of enzyme-linked immunosorbent assay.

result of this strategy is the ability of antibodies, and the resulting assays, to distinguish between enantiomers of chiral drugs (7). Although coupling methods generally have involved condensation reactions of hapten carboxylic or amino groups (or activated derivatives thereof) with amino or carboxylic groups on proteins, newer conjugation methods continue to be developed. These include the use of two-level, heterobifunctional agents such as *N*-(*m*-aminobenzoyloxy) succinimide, first to form a peptide bond to carrier protein and then, following diazotization of the aromatic amino group, to couple to a suitable hapten containing an imidazole, phenol, or indole residue. An example is the coupling of thyroid stimulating hormone to BSA (8). Another example involves interesting steroid chemistry in the preparation of an 11-Alpha-(3-Sulfanylpropyl)oxy hapten analog of the 3-Sulfamate ester of estradiol, and its subsequent coupling to bovine gamma globulin via a heterobifunctional crosslinker (9).

IMMUNOASSAYS FOR MACROMOLECULES

Immunoassays for macromolecules generally fall into one of two categories, namely those for endogenous proteins applied in support of clinical medicine (for example, assays for gonadotrophins or insulin) or those for new, genetically engineered proteins. The advent of genomics, proteomics, and recombinant technology has greatly advanced our understanding of the potential roles of regulatory proteins in the pathogenesis and modulation of diseases, and led to a sharp increase in the number of biological therapeutics under development. Several regulatory proteins are being developed as therapeutic agents and some have reached the market. Fig. 4 indicates the wide distribution of these agents across many different therapeutic categories, with a concentration in the field of cancer treatment. These products are often recombinant analogs of endogenous proteins, with the resulting challenge of developing enzyme immunoassays that are specific enough to distinguish between native and recombinant molecules.

Unlike low-molecular-weight xenobiotics, macromolecules are often immunogenic. An immune response is often elicited against several sites on the molecules called epitopes or antigenic determinants. The number of epitopes per antigen is determined by the size and complexity of the molecule. These epitopes can be linear, consisting of as few as four amino acids in a sequence, or conformational, involving different regions of the molecule in a three-dimensional configuration. Use of antibodies against conformational epitopes can be problematic if the antigen of interest loses its three-dimensional structure in vivo, or during sample analysis. In general, assay formats for macromolecules use at least two antibodies that react with two different regions of the molecule. Use of two antibodies against different regions of the molecule can confer additional specificity and may distinguish the analyte of interest from its metabolites or isoforms.

IMMUNOASSAYS FOR ANTIBODIES

Many of the new biological agents under development are recombinant versions of naturally occurring human proteins, or analogs of human proteins containing minor changes in their primary sequence, or differences in the extent of post-translation modifications. Marketed recombinant proteins include hormones (insulin, erythropoietin and growth hormone), enzymes (DNAase, asparaginase), cytokines (interleukins 1, 2, 11, interferon), growth factors

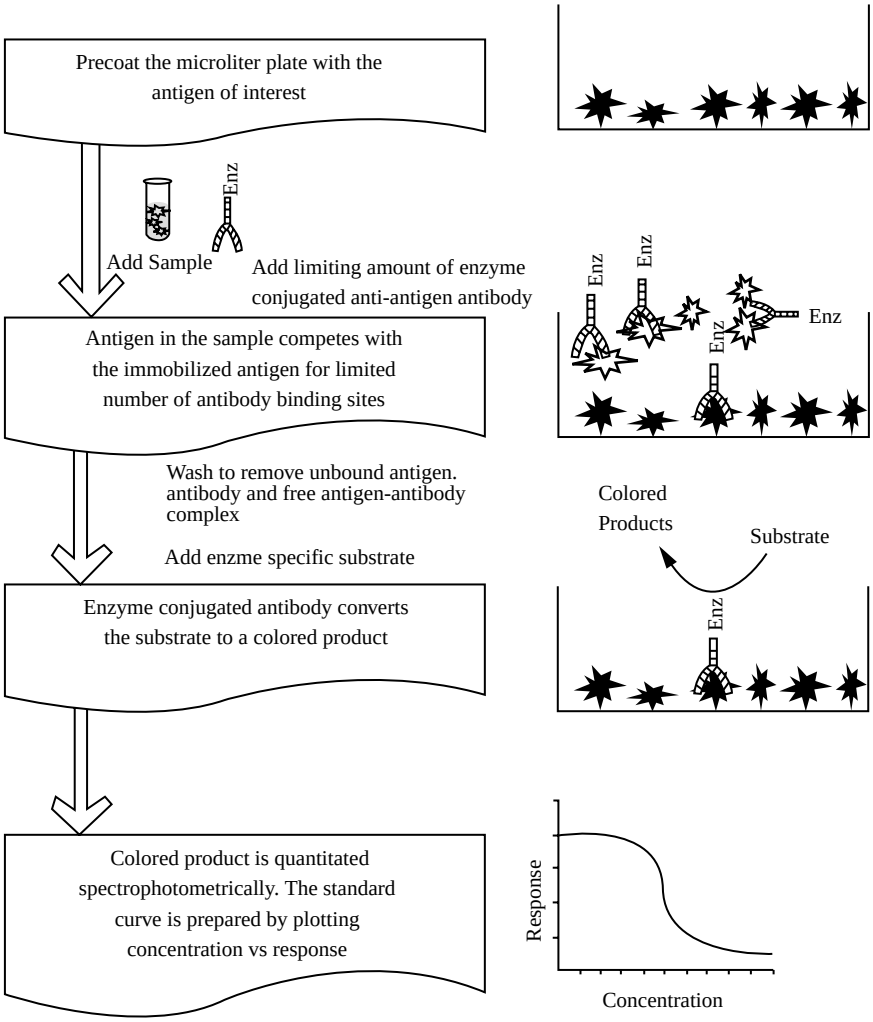

Fig. 3 An illustration of competitive enzyme-linked immunosorbent assay.

(G-CSF, GM-CSF), clotting factors (Factor VIII) and vaccines (hepatitis B). Some other biotechnology products are novel fusion proteins, such as etanercept (Enbrel®), whereas yet others are therapeutic antibodies, for example muromonab (Orthoclone OKT3), abciximab (ReoPro®), and trastuzumab (Herceptin®). Administration of these recombinant proteins to animals and humans may result in their recognition by the host's immune system as "non-self," resulting in an antibody response. Several factors contribute to the potential immunogenicity of these molecules, including the structure of the protein (including post-translation modifications), the presence of protein fragments or protein aggregates in the administered formulation, and the cell substrate or media components that may co-purify with the therapeutic agent. Clinical factors, such as genetic background, disease state or immune status, may also influence the immunogenicity of a biological product, as well as the route and frequency of dose administration.

In both preclinical and clinical studies, evaluation of immune response to the administered product is necessary to evaluate accurately the safety, pharmacokinetic and pharmacodynamic response as anti-drug antibodies can bind the drug and neutralize the therapeutic effect, or eliminate it by-Fc receptor-mediated uptake and destruction in the reticuloendothelial system. Conversely, the pharmacodynamic response could be enhanced if the distribution or clearance of the drug–antibody complex is altered (10). Antibodies to therapeutic agents may also react with the endogenous analog protein, abrogate its activity, and precipitate a severe adverse event. The presence of such antibodies may also interfere with the immunoassay for the quantitation of the therapeutic agent in biological matrices. In addition, the presence of

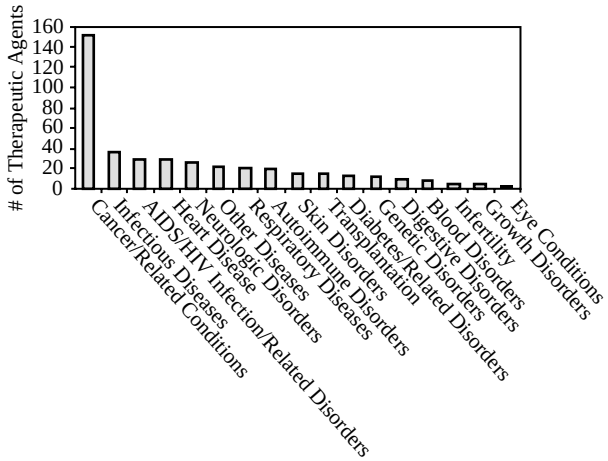

Fig. 4 Biotechnology products under development. (From New Medicines in Development, Biotechnology, PhRMA, April 1998.)

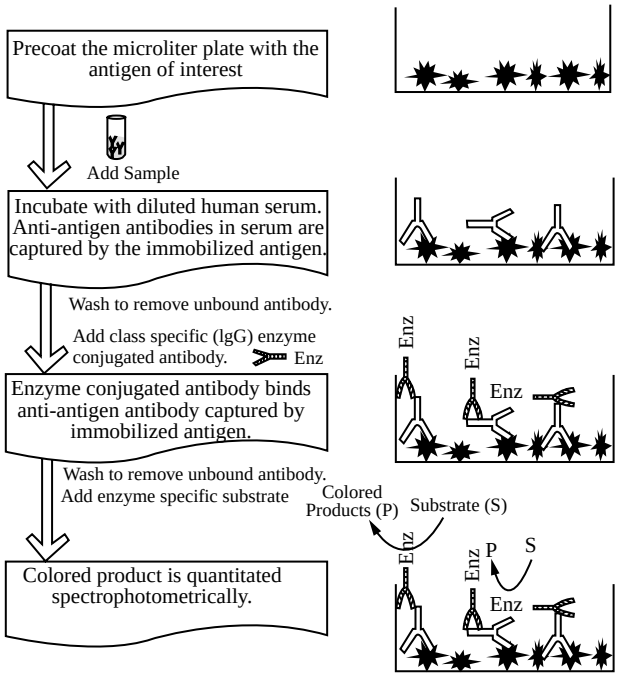

Fig. 5 An illustration of antigen capture antibody detection assay.

pre-existing antibodies (autoantibodies) to endogenous proteins can further complicate the quantitation of the molecule in biological fluids and its safety assessment in preclinical and clinical studies. Anti-drug antibodies may also interfere in imaging and diagnostic procedures utilizing antibodies; for example, human anti-mouse antibodies (HAMA) in the serum of patients treated with a murine antibody-based therapeutic may interfere in diagnostic assays using murine monoclonal antibodies.

Although several immunoassay formats [precipitin reactions, agglutination, radioimmunoassay (RIA), immunoradiometric assay (IRMA), western blot] have been used to detect and quantitate antibodies, enzyme immunoassay is the most commonly applied method. Several factors should be considered when developing an assay to detect the antibody response to a therapeutic agent. It is essential to understand the purpose for the antibody detection assay as it will influence the selection of the assay format. If the aim of the work is to detect only high-affinity antibodies with concomitant high specificity, a competitive assay format would be most appropriate. However, if all antibodies to the administered molecules (regardless of their affinity) are to be detected, best results will be obtained with a noncompetitive ELISA. In addition, random orientation of the antigen to expose all potential epitopes should be confirmed during assay development. These formats are illustrated in Figs. 5, 6. Product characteristics, such as the impurity profile, protein structure, and the presence of fragments and aggregates of the administered protein, need to be understood so that the most appropriate antigen is used in the assay. The potential loss of epitopes when the protein is directly adsorbed on the surface of the plate, the possibility of circulating antigen–antibody complexes, circulating antigen aggregates, and the source species of antibodies for capture and detection in the assay should also be evaluated. In some instances, cross-reactivity of the detected antibody with subclasses of the macromolecule (e.g., interferon) may need to be evaluated because the binding affinities of each subtype may vary greatly and may influence the interpretation of the results. Pre-existing antibodies can also limit the application of another antibody raised in the same species as that used in the assay configuration (e.g., as the detection antibody). Concomitant medications or high levels of the circulating macromolecular therapeutic agent may also be potential interferents in the assay.

Whether these assays should be established as truly quantitative is controversial. Although a quantitative response seems, from a bioanalytical perspective, desirable, the difficulties in so establishing the assay are significant and the added value is debatable. The primary challenge in developing a quantitative antibody assay is the lack of well-characterized, species-specific, polyclonal anti-drug antibody reference materials to be used as calibration standards (11). Heterologous polyclonal antibody could be used as reference standard (e.g., monkey antibody for human studies), or even nonspecific human IgG. However, although a true quantitative titer

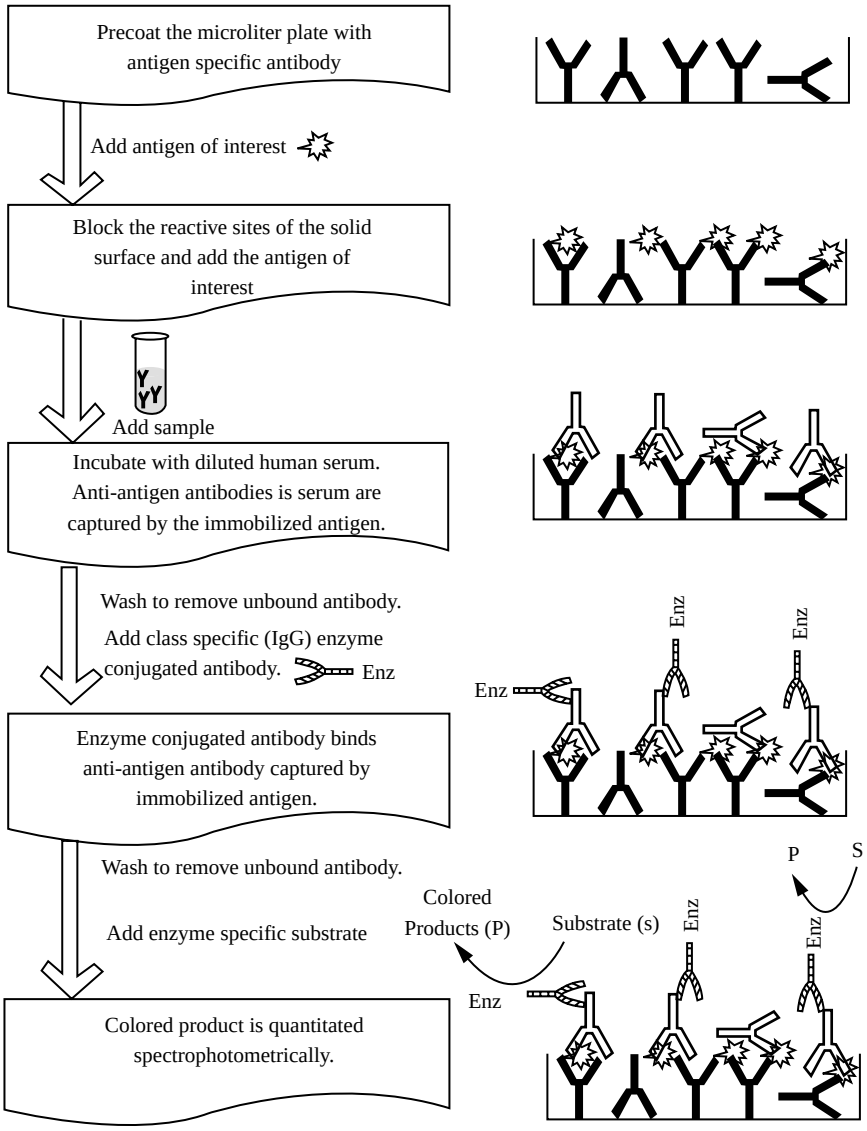

Fig. 6 An illustration of antibody detection method using an antigen–antibody capture configuration.

value may be calculated with reference to a standard of one of these types, the greater value of ELISA assays for antibodies to macromolecules lies in comparative titers determined over time following administration of the candidate therapeutic protein, in conjunction with the assessment of any clinical sequelae.

IMMUNOASSAYS FOR BIOMARKERS

A biomarker is defined as an in vivo biological response to a disease or a toxicological event. Pharmacological markers are a subset of biomarkers that respond to drug intervention, whereas surrogate markers are markers that predict clinical endpoints. In a clinical setting, biomarkers may indicate the response of a disease to therapeutic intervention. In the pharmaceutical industry, correctly chosen biomarkers may help with compound selection, identify mechanism of action, predict dose, demonstrate efficacy and, in cases where sufficiently sensitive assays are unavailable, substitute for direct pharmacokinetic evaluation. Identification of relevant biomarkers at the discovery stage will optimize target selection and validation, facilitate preclinical toxicological evaluation, and markedly shorten the time needed to demonstrate proof of concept in early clinical trials. Rapid and effective implementation of appropriate biomarker assays will result in major resource

savings in drug development. A biomarker may be a physiological measurement, such as blood pressure or heart rate, an enzyme activity, or a quantifiable discrete molecule. Biomarkers can be either low-molecular-weight analytes or large macromolecules. Many of these molecules (especially macromolecules) are measured by immunoassay, particularly by enzyme immunoassay.

Many biomarkers are found in blood and urine, where the basal levels may be low or high, depending on the nature of the marker. Thus, one of the additional challenges in establishing enzyme immunoassays for these analytes is obtaining analyte-free matrix for use in preparation of calibration standard and quality control samples. Appropriate analyte-free matrix may sometimes be prepared by extraction of the analyte from matrix by such techniques as affinity chromatography (6) or charcoal adsorption, but care must be taken to ensure that the processed matrix is still representative of the matrix to be analyzed. Alternative solutions to this problem include use of the same matrix type from a different species in which the endogenous biomarker does not occur, pooled baseline samples of low biomarker concentration, or use of a protein-containing buffer. In each case, dilutional linearity of study samples, containing high concentration of the analyte, with the assay matrix should be demonstrated. However, although calibration standards may be prepared in an alternative matrix, whenever possible the quality control samples should be prepared in the matrix to be analyzed so that they reflect the assay performance for the study samples. The concentration range for the calibration standards should attempt to bracket the anticipated concentrations of the biomarkers in both physiological (including such factors as circadian rhythm and intra-individual variability) and pathological states. The concentration levels in the quality controls should also reflect concentration levels in physiological and pathological disease states. Physiological levels may be affected by gender, race, intra-individual variation, circadian rhythm, and seasonal variations, whereas the levels in pathological samples may be affected by stage of the disease, intercurrent disease, current therapies, and overall patient status.

Another challenge in developing assays for biomarkers is the inconsistent availability of well-characterized reference standards. For many macromolecular biomarkers, purified species-specific reference materials are not available. If available, proteins are often not characterized by a standardized method. Different sources of antibodies may also give different results for the same lot of reference standard. It is suggested that crossover studies using standards, quality control pools, and some study samples should be conducted when the source or lot number of reference standard or antibody is changed. The data from crossover studies will help to normalize study results obtained with different lots of reagents. In addition, biomarkers may exist in multiple forms, such as isoforms, or have homology with other biomarkers in the same class of molecules. Thus, an immunoassay may be developed to measure all forms nonspecifically or target a specific isoform. Cross-reactivity of the antibody selected for use in a biomarker assay should be rigorously tested for potential cross-reactivity with other isoforms and homologous molecules.

VALIDATION OF ENZYME IMMUNOASSAYS

Validation of bioanalytical methods has been a subject of increasing attention over the last 10–15 years. A conference addressing this issue, cosponsored by the US Food and Drug Administration (FDA), the Health Protection Branch (HPB) Canada, the American Association of Pharmaceutical Scientists (AAPS), the European Federation of Pharmaceutical Societies and the United States Pharmacopoeia (USP), was convened in Arlington, Virginia, in December 1990. The proceedings of this meeting, which came to be known as the Crystal City meeting, were published (12) and have subsequently been used as an informal guideline for bioanalytical method validation.

Since 1990, bioanalytical method validation has been a topic of discussion at the International Conference on Harmonization, and was also the topic of a draft guidance document from the FDA (13). Although, many of the validation considerations for chromatographic assays also apply to immunoassay validation, some of the unique considerations for immunoassays were not addressed in the draft guidance. The present authors, along with a number of pharmaceutical industry colleagues, have recently discussed validation issues specific to immunoassays (14). Topics covered in that review included the proper use of quality control (QC) samples for acceptance of assay runs, and statistical aspects of assay validation. Specifically addressed was the issue of how differences in bioanalytical techniques should be considered when developing validation acceptance criteria. In recent years, several publications have reviewed issues related to validation of immunoassays both broadly (15, 16), and specifically for assays for macromolecules (17). In 2000, two conferences were held to "revisit" the issue of bioanalytical methods validation in general (18), as well as specifically for assays for macromolecules, primarily immunoassays and cell-based assays (19).

As for any bioanalytical method, the extent of validation for an immunoassay should be related to the

intended application of the assay. Thus, if an immunoassay is intended to support rapid screening in discovery R&D, the characterization of specificity and the accuracy and precision specifications may be less stringent than if the assay is used to support preclinical and clinical development studies. Indeed, an assay for discovery support may be designed to detect active metabolites as well as parent molecule, so that an estimate of total, circulating, pharmacologically active agents may be made. However, at the development stage, such an assay may be applicable only with clear definition of the cross-reactivities of both the parent and the active metabolite in the assay.

DIFFERENTIATING CHARACTERISTICS OF IMMUNOASSAYS AND THEIR IMPACT ON VALIDATION

The key difference between chromatographic assays and immunoassays is the biological nature of the critical binding reagent in an immunoassay, namely the antibody. As antibodies are produced in biological systems, lot-to-lot variability may occur, and this is greatest for polyclonal antibodies because they are produced in whole animals. Monoclonal antibodies, produced from a single cell line in an in vitro biological system, tend to have much lower variability between different production batches. Immunoassays have at least one timed incubation period, which means that variations in binding affinity and avidity between different lots of antibody reagents can result in differences in rates at which equilibrium is reached. Another important difference between immunoassay and chromatographic assays is the use of analyte–protein and antibody–protein conjugates in immunoassays. During validation, the stability of these critical reagents should be demonstrated to ensure that their degradation does not adversely affect assay performance. The availability of reference standards may also differentiate chromatographic and immunoassay procedures. Thus, although well-characterized reference standards are readily available for low-molecular-weight xenobiotics, it is sometimes much more difficult to obtain similarly well characterized reference materials for macromolecule immunoassays. Macromolecular products are not always available in a highly purified state, and are often characterized in terms of biological activity rather than percentage purity. In some cases, more widely studied proteins are available as reference standards from independent agencies such as the World Health Organization.

Another important difference between immunoassays and most chromatographic assays, with significant implications for validation, is the nonlinear nature of the relationship between concentration and response for immunoassays. For optimum calibration curve fit, it is sometimes appropriate to include calibration points above and below the defined limits of quantitation. The calibration curve fit algorithm should evaluate the overall fit of the experimental data with and without the use of these additional standards in the asymptotic regions of the calibration curve. In general, the authors have observed that the use of additional standards in the asymptotic regions improves the accuracy and precision of the assay at the limits of quantitation. The option to include additional standards is not addressed in the draft FDA guideline for bioanalytical method validation (13), which states that the lowest and highest concentrations on the calibration curve should serve as the limits of quantitation.

Precision, accuracy and specificity also raise some interesting and different considerations for immunoassay methods. The biological nature of the reagents and the antibody–antigen reaction can potentially confer higher imprecision on immunoassays, and a larger number of validation runs may be needed to determine the true precision of the method. This higher imprecision also increases the likelihood that a higher number of assay runs will not meet the so called 4-6-20 rule relating to quality control acceptance criteria for method implementation (20, 21), leading to a recommendation (14, 19) that the 20% limit for accuracy be relaxed to 25% for immunoassays. However, as many immunoassays can also have precision comparable to that of chromatographic methods, the acceptance criteria should be determined based on the demonstrated capability of the method during validation and not set arbitrarily to 4-6-25 for all immunoassays.

Although antibodies can be exquisitely specific, the biological nature of these reagents also poses some new specificity issues for immunoassays when compared to chromatographic assays. In immunoassays, the analyte of interest is usually detected and quantitated directly (i.e., without prior extraction) in complex biological matrices such as serum, plasma, or urine. Furthermore, the specificity of the assay can potentially be compromised if cross-reacting metabolites of the analyte of interest are present in the study sample. Nonspecificity in immunoassays can arise from a variety of sources, but may be broadly classified as "specific" or "nonspecific" nonspecificity. Specific nonspecificity can arise from interferents that share similar physicochemical characteristics with the analyte of interest, and include metabolites, degraded forms of the analyte, impurities, or concomitant medications. For macromolecules, post-translation modified proteins,

protein aggregates or host anti-idiotypic antibodies may affect the specificity of the assay in a "specific" manner. Nonspecific nonspecificity arises from a variety of factors unrelated to the analyte, and is often referred to as "matrix effects." Matrix effects may be due to hemolysis, lipemia, ionic strength differences, pH, serum proteins such as complement or rheumatoid factor, anticoagulants, binding proteins, autoantibodies or heterophilic anti-IgG antibodies. Nonspecific interferences may arise from the matrix chosen for preparation of calibration standards and quality control samples, or the study sample itself. Evaluation of the matrix should include comparison of the concentration–response relationship in spiked (and unspiked) matrix to that in a buffer matrix. Dilution with buffer may adequately decrease the intensity of matrix effects; however, if this approach fails, sample cleanup or full analyte extraction from the matrix may be needed. In these cases, it is important to treat all samples, including calibration standards, quality control or validation samples and study samples, identically, to obviate the need for recovery corrections in the assay.

VALIDATION CONSIDERATIONS FOR IMMUNOASSAYS FOR LOW-MOLECULAR-WEIGHT XENOBIOTICS AND MACROMOLECULES

An essential prerequisite component of small-molecule immunoassay validation is the demonstration of specificity for the analyte of interest. Assay interference (cross-reactivity) due to known metabolites, concomitant medications and, in some cases, endogenous molecules, should be evaluated. These experiments should assess the cross-reactivity of the potential interferents individually and in combination with each other and the analyte of interest to simulate the most likely biological milieu for the analyte. For small molecule immunoassays, the specificity of the assay should be established, whenever possible, by conducting a comparator study over a specified number of analytical runs, using a different, validated and specific bioanalytical method, such as LC–MS. Samples for these comparative analyses should be selected from an earlier study (incurred samples), and should have been collected at two or more time points following drug administration (e.g., approximate time of maximum plasma concentration and a succeeding time corresponding to several elimination half-lives) to allow performance of the immunoassay to be evaluated in an environment of increasing metabolite(s) concentration. If the immunoassay meets the predefined criteria for accuracy and precision relative to the reference method, the immunoassay may be considered equivalent to the reference method.

The metabolism/catabolism of protein drugs and protein drug candidates is generally much less clearly elucidated than that of conventional small-molecule therapeutics. The dearth of information regarding the metabolism of protein-based drug candidates can hamper efforts to develop a highly specific and accurate enzyme immunoassay. In addition, there are few, sufficiently sensitive, comparator methods available to perform comparative analysis. Approaches to define specificity and, thus, predict reliability of a given assay, include epitope mapping experiments or the use of chromatographic separation prior to immunoassay, although the latter approach can be cumbersome and may result in markedly reduced assay sensitivity. Consequently, complete investigation of assay specificity for a therapeutic protein is more difficult than for a low-molecular-weight xenobiotic. The immunoreactivity of the analyte may be decreased with relatively minor changes in the region of antigenic determinants, such as a change in the amino acid sequence or by oxidation/deamination of an amino acid. On the other hand, proteolytic formation of major fragments of the parent protein may result in retention of the antigenic determinants and preserve the immunoreactivity of the protein metabolite. Such structural changes may or may not result in changes in biological activity, so that immunoreactivity may or may not correlate with biological activity in the study samples collected after administration of the protein. If assay sensitivity permits, methods such as chromatography or electrophoresis, coupled with immunoassay, may shed some light on the structural nature of the compounds in the study samples giving a positive response in the immunoassay. For many recombinant proteins, antigenic determinants of the therapeutic agent are often indistinguishable from those of the endogenous equivalent protein and, therefore, assays for such recombinant proteins are prone to interference from endogenous analog proteins. Such an occurrence poses challenges for the validation analyst in the selection of the preferred homologous matrix for the preparation of calibration standards, validation pools and quality control samples. The problem is accentuated when the administered doses of the exogenous product are so low that circulating concentrations are not increased markedly over the background endogenous concentrations. Clearly, in cases where in vivo concentrations of the exogenous product are very high after dosing, the contribution of low basal levels of the endogenous analyte to the total measured concentration may be small enough to be ignored. An alternative approach to this problem is to remove the endogenous analyte from the matrix by one of a number of methods, as discussed previously (under the heading

"Immunoassays for Biomarkers") or to use a heterologous biological matrix devoid of the specific interfering endogenous substance for the preparation of calibration standards and control samples. In all of these approaches, it is important that the final prepared calibration curve reflects negligible bias due to the presence of endogenous analyte.

VALIDATION CHALLENGES FOR ENDOGENOUS ANALYTE AND BIOMARKER IMMUNOASSAYS

Immunoassays are often developed for the quantitation of endogenous equivalents of therapeutic molecules and biomarkers. Challenges in developing immunoassays for these compounds are similar to those experienced with macromolecules. As the biomarker is normally always present in the matrix of interest, it is difficult to obtain analyte-free matrices. Standard curves and lower limit of quantitation (LLOQ) validation pools may be prepared by choosing and pooling matrix from individuals with low baseline concentrations, diluting baseline samples with a protein-based buffer, or using an alternative species matrix with negligible concentrations of the analyte. The upper limit of quantitation (ULOQ) can be established by fortifying the baseline sample with the analyte of interest. Whenever possible, the appropriate biological matrix should be used for QC sample preparation. This may be a systemic matrix such as whole blood or plasma, or target-specific matrix such as sputum, cerebrospinal fluid, aqueous humor, platelets, T-cells or tissues. Alternatively, if no matrix effects can be demonstrated, quality control pools may be prepared in an "analyte-free" protein-based buffer.

As with macromolecules, obtaining well-characterized reference material can be difficult. Whenever possible, reference materials should also be species-specific. In situations where well-characterized standards are not available, crossover studies should be conducted to permit normalization of data obtained using reference standards from different vendors or different lots.

VALIDATION CONSIDERATIONS FOR IMMUNOASSAYS FOR ANTIBODIES

Anti-drug antibodies are polyclonal in nature and rarely does one have access to species-specific (especially human) anti-drug antibodies to prepare a calibration curve. Despite the problems associated with developing a quantitative anti-drug antibody assay, most validation parameters for immunoassays still apply. Although true accuracy cannot be determined, relative recovery using quality control samples can be monitored through the life of the assays. Assay specificity evaluation should include assessment of any nonspecific binding of the antibody to the microtiter plate, potential interference of the administered protein, endogenous protein analogs, concomitantly administered drugs, and antigen–antibody complex or cross-reacting antibodies that may be present in the sample under evaluation.

A major issue with anti-drug antibody assays is definition and interpretation of a positive antibody response. During validation, a negative cut-off value, to distinguish antibody negative and positive results, should be determined by evaluating the analytical noise (imprecision) of the assay and the background absorbance readings from individual baseline samples from healthy volunteers and patients from the appropriate disease population. The recommended number of baseline samples is at least 25 (preferably 100) from each of the volunteer and patient groups. If the background responses tend to cluster closely together and the assay is precise, a negative cut-off can be defined as the mean absorbance ± 3 SD at a given dilution factor(s). If, however, there is greater variability in the background absorbance, developing a negative cut-off is more difficult. The cut-off should be based on an acceptable level of false negative and false positive results. The assay validation scheme should include a process to distinguish true responses from false positives. This is particularly important as auto-antibodies against various proteins may be present in otherwise healthy individuals. Several approaches can be taken to elucidate whether an apparent antibody response is truly positive, such as an alternative method for detecting the antibody (e.g., western blotting). In some cases, when antibody response is evaluated against several different antigens, true positives may be distinguished from false positives by cross-reactivity patterns. Finally, examination of the response prior to drug administration, and the change in this response over time, with continued exposure to the agent, will normally distinguish between true and false positives. In addition, results of in vitro neutralizing activity assays and clinical effects (in vivo neutralization) may provide further support for the presence of clinically significant, drug-specific antibodies.

MODERN TECHNOLOGICAL ADVANCES RELATED TO ENZYME IMMUNOASSAY

Immunoassays are inherently sensitive and specific. However, with continued need to develop increasingly sensitive assays to support preclinical and clinical

studies, there have been ongoing efforts to enhance the capabilities of these techniques. Advances in critical binding reagents, detection systems, new assay formats and automation have resulted in improved immunoassay technology.

Critical Binding Reagents

Although most immunoassays have used polyclonal antibodies as the critical binding reagents, development of monoclonal antibodies by Kohler and Milstein in 1975 (22), has resulted in their widespread use, particularly in assays for macromolecules. Their unique epitope specificity conveys advantages in double antibody immunoassays for proteins, where one monoclonal antibody may be used to capture the protein by a specific subunit or epitope, and another, directed against a different region or subunit of the protein, may be used to detect it. Use of antibodies against specific regions of the molecule can enhance the specificity of the assay such that one can distinguish the parent molecule from its catabolic products, one isoform from another, or an individual protein from its family members. Although monoclonal antibodies are highly specific, their affinity for the antigen is generally lower than that observed with polyclonal antibodies. Consequently, competitive immunoassays established with monoclonal antibodies as the critical binding reagent are generally less sensitive than those using polyclonal antibodies. For this reason, the application of monoclonal antibodies to competitive immunoassays for low-molecular-weight analytes has been limited.

A nonantibody binding reagent that has received increasing attention recently is the aptamer. Aptamers are oligonucleotide sequences that bind ligands or antigens in a way that is similar in many respects to antibody–ligand interactions (23). Thus, aptamers have been shown to bind with high affinity and selectivity to molecules as diverse as proteins and low-molecular-weight ligands. Aptamer libraries have also been generated, from which members with the desired binding properties may be identified and their concentrations enriched for use in binding assays. The use of these molecules as complements to, or substitutes for, antibodies has been reviewed elsewhere (24). These reagents offer the promise of similar sensitivity and specificity achievable with antibodies, without the need for time-consuming in vivo work to generate them.

Phage display technology provides a source of recombinant antibodies with defined affinity and specificity for use in immunoassay, without the need for extended immunization of animals. This approach involves genetic manipulation of the coat proteins of the filamentous phage, a bacteriophage that lives on *Escherichia coli*. In one approach, the coding sequences of the antibody variable regions (Fv) are first isolated from spleen cells of immunized mice. The coding sequence for a single chain Fv fragment is then fused to the phage coat protein. With the assistance of a series of molecular biology steps, a library containing millions of single-chain antibodies can be displayed on the surface of the phage particles and released into the medium. Through a series of binding and elution steps ("panning"), the mixture of antibodies with the desired affinity and selectivity may be sequentially enriched. To obtain single-chain antibodies of suitable affinity and selectivity against new drug entities, one can repeatedly and rapidly screen the recombinant antibody phage library and avoid the traditional, time-consuming process of antibody production. A detailed discussion of antibody phase display technology is provided in reviews by Hoogenboom (25) or Peterson (26).

Molecularly imprinted polymers (MIPs) also offer some potential as synthetic alternative binding reagents (27) for assay of small molecules. These binding reagents are synthesized by polymerization of functional monomers (e.g., methylacrylic acid, 4-vinyl pyridines) in the presence of the ligand (antigen), which acts as a template. Depending on experimental conditions during polymerization, the template ligand may interact with the monomers by either noncovalent interactions, reversible covalent interactions, or metal-ion-mediated interactions, with the noncovalent approach being most commonly used. Upon completion of the polymerization reaction, the ligand may be washed out to leave its imprint in the polymer. The binding properties of these molecular imprints are characterized by remarkable specificity for the ligand originally imprinted. These MIPs have been applied in place of biologically derived antibodies for the binding assay of a range of low-molecular-weight analytes following extraction of the analyte from the biological matrix. The assays were initially conducted in an organic solvent. Under these conditions, a good correlation was found between the MIP binding assay and an established radioimmunoassay for theophylline (28). Subsequent developments of aqueous assay conditions led to a MIP-based assay for propranolol directly in plasma (29). These high-affinity binding reagents have high chemical and thermal stabilities, resulting in long shelf lives at ambient temperature, an advantage over antibodies. Additionally, although most MIP-based assays have employed radiolabeled analyte as the detection system, some recent studies have successfully used a fluorescence

detection system. However, there are currently some limitations with this technology. The MIPs often have lower binding affinities than do antibodies, resulting in lower assay sensitivities than immunoassays have. The use of MIPs is also limited to analytes stable in organic matrices, and further research is necessary to establish them fully in aqueous media, so that they are competitive with conventional immunoassays for the direct analysis of biological fluids.

Detection Systems

The need for greater sensitivity to monitor extremely low concentrations of either highly potent therapeutic agents, endogenous biomarker molecules, or environmental toxicants has been the primary driver in the development of newer detection systems as more sensitive alternatives to the chromogenic substrates normally used in enzyme immunoassay.

There are numerous examples in the literature of fluorescence being used in place of ultraviolet light absorption as the end-point detection system for an immunoassay. In particular, methods using time-resolved fluorescence detection (30) offer high sensitivity while largely avoiding the problem of background fluorescence in complex matrices by allowing this short-lived fluorescence to decay, before fluorescence of the labeled antibody complex is measured. These procedures frequently employ lanthanide chelates as the long-lived fluorophores. An interesting recent example of this method is the time-resolved fluoroimmunoassay for plasma enterolactone, a lignan produced from fiber-rich foods by intestinal bacteria, and thus claimed to be a biomarker of a healthy diet (31). This assay used a derivative of enterolactone coupled with a europium chelate as the fluorophore, and achieved sensitivity of 1.5 nmol/L. Examples from the pharmaceutical field include the assays for enalaprilat in human serum (32) and sampatrilat in human plasma (33), which are characterized by lower limits of quantitation of 200–500 pg/mL. In the technique of fluorescence polarization, detection is based on the change in polarization of light emitted by a fluorophore molecule when bound to an antibody. This change in polarization is correlated with the concentration of unlabeled antigen, and a standard curve is developed to interpolate the analyte concentration in an unknown sample. This method, which has the advantage of being homogeneous and easily automatable, has been widely available commercially for some time for such applications as therapeutic drug monitoring, but more recently has seen new application to high throughput screening in drug discovery (34).

Chemiluminescence (35) offers yet another sensitive detection system, which is easily implemented with simple instrumentation, but suffers to some extent from background interference in complex matrices. A recent example of an enzyme immunoassay with chemiluminescence as the detection system is the assay for 8-oxoguanine in DNA (36), which uses a secondary antibody conjugated with peroxidase–anti-peroxidase complex and a substrate solution containing hydrogen peroxide, luminol and *p*-Iodophenol.

The utility of chemiluminescence as a detection system has been extended greatly with the development of electrochemiluminescence. Although electrochemiluminescence has been studied since the 1960s, only relatively recently has the system been commercialized by the IGEN Corporation (Rockville, MD). In their system (37), a precursor molecule, tripropylamine (TPA) diffuses to an electrode surface to be activated, resulting in the excitation of a reporter molecule, ruthenium *tris*-bipyridyl. When the reporter molecule returns to ground state from the excited state, it emits a photon of light at a specific wavelength, which is detected by a sensitive photomultiplier tube. The system includes an electrochemical flow cell and magnetic bead technology to trap the ruthenium-tagged molecules on the electrode and thus allow the electrochemical cycle to proceed. Thus, in a typical immunoassay for a macromolecule, two different anti-analyte antibodies (recognizing different epitopes) may be used. One antibody may be labeled with biotin, which complexes with strepavidin-complexed magnetic beads, whereas the second antibody is labeled with the ruthenium complex. During incubation, the analyte is sandwiched between the two antibodies, after which the mixture is drawn into the flow cell, and the antigen–antibody complex is trapped on the electrode surface by magnetic forces. After washing, the amount of ruthenium complex in the trapped antigen–antibody complex is measured by activating the electrode and quantitating the emitted light. This method has been applied to bioanalysis of many analytes; an example is the biotin–avidin coupled assay for interferon alfa-2b in human serum, with a sensitivity of 4 IU/mL (38).

Detection systems have extended into the area of biosensors and immunosensors, with the application of surface plasmon resonance (SPR) in immunochemistry. SPR is one of a number of optical immunosensor techniques (39) in which a change in the resonance angle of incident light occurs when antigen-antibody binding takes place. In an instrument such as the Biacore, a typical experimental design might involve adsorption of an antibody to the gold or silver surface of a microcell, which is backed by a prism or diffraction grating. When a solution containing the antigen of interest flows through

the cell, the formation of the antigen–antibody complex results in a change in the angle of the reflected light (resonance angle) at the metal surface. The shift in the resonance angle has been reported to have a linear relationship to the concentration of antigen added to the system. As no labeled reagents are needed for this method, SPR can be quite simple; however, the technique cannot distinguish between antigen recognition and nonspecific binding, and poor sensitivity can also be a limiting factor. However, recent advances include the incorporation of liposomes linked to a sandwich immunoassay format, resulting in picomolar sensitivity in an assay for interferon (40). In another application for a low-molecular-weight xenobiotic, sulfamethazine, in milk, the analyte was covalently coupled to the gold surface of the sensor chip (41). The final response was the result of competition between covalently bound sulfamethazine and free antigen in calibration solutions and study samples for binding sites on polyclonal antibodies, also in solution. The assay sensitivity was in the range of 1.7–8.0 μg/kg.

Assay Configuration and Automation

When immunoassay specificity has not been inherent in the antibody employed in the assay, separation steps such as high performance liquid chromatography have been applied prior to immunoassay. One of the more promising of such coupled methods is capillary electrophoretic immunoassay (CEIA) (42). This method offers a number of potential advantages, including a smaller sample size and lower reagent consumption, simple and readily automatable process, potential for simultaneous determination of multiple analytes, and a broad range of detection techniques. When coupled with laser-induced fluorescence as the detection method and enzyme amplification, CEIA appears to be competitive with standard immunoassay techniques, with assay sensitivity in the 10-pM range. The resolving capabilities of CEIA can separate antibody-bound from free antigen, followed by application of the detection method. Thus, the technique is configured for on-line application. Although clearly having the potential for high throughput, CEIA has been applied only in a serial mode to date, analyzing one sample at a time. CEIA has been applied widely to the characterization of antibodies, as well as to immunoassay of a number of low-molecular-weight analytes, including digoxin, morphine and cortisol.

Immunoassay has achieved considerable success in the medical diagnostic arena largely due to its facile adaptation to automation, high sample throughput and relatively low per sample cost. Issues and challenges involved in the automation of immunoassays have been addressed recently by Bock (43). An interesting recent application of enzyme immunoassay in an automated mode has been in the support of high throughput screening in drug discovery (44). In this case, the assay reagents are incorporated into a gel matrix rather than in a multiwell format, which permits 1,000–10,000 assays to be run per day, with the assistance of automation, by a single technician. The development of multianalyte immunoassays in miniaturized, microarray formats has also been reported (45).

The move toward automation has also led to development of online and flow injection immunoassays. These methods involve sequential injection of assay reagents and antigen–antibody reactions in flowing systems. One such system with some promise is flow injection renewable surface immunoassay (FIRSI), with fluorescence detection (46). In this flowing system, antibody-coated beads are retained on a flat surface adjacent to the detector; labeled and unlabeled analyte are then injected and flow over the beads, while reaction occurs. The beads are then washed, the final antibody-bound reading occurs at the detector and the flow is reversed to remove the beads in preparation for the next injection. Similar systems, sometimes using magnetic particle-coupled immunoglobulin to facilitate the separation of antibody-bound and free ligand (47), have been used in conjunction with electrochemiluminescence (48) or laser-induced fluorescence (49) as the detection methods.

CONCLUSIONS

Enzyme immunoassay is widely used, both in competitive and noncompetitive formats, for the bioanalysis of a broad range of low-molecular-weight compounds and macromolecules. Through the use of fluorogenic substrates and amplification systems such as avidin–biotin, the sensitivity of enzyme immunoassay has been developed to equal or exceed that of radioimmunoassay (50). The technique has found particularly wide applicability in the determination of new recombinant proteins, in demonstrating antibody responses to macromolecules, and in the measurement of biomarkers of disease, as well as in diagnostic medicine.

As for all bioanalytical methods applied to support of drug development, validation of immunoassays is important. However, several validation issues need special attention for immunoassays. These include stability of the critical reagents, the curvilinear nature of the calibration curve, the greater variability of immunoassays, and, particularly important, the specificity of the assay.

Finally, a number of newer binding reagents, detection methods, assay configurations, and automation applications are being investigated to develop further the potential of immunoassays. These include binding reagents requiring little or no animal immunization for their production, such as phage display antibody libraries, aptamer libraries, and synthetic molecular imprints. Detection methods such as electrochemiluminescence and surface plasmon resonance hold promise of sensitivity equal to, or better than, that of radioimmunoassay without the limitations of radioactivity. Progress has also been made in automation and miniaturization of immunoassays, as well as online techniques, such as CEIA and flow injection methods. These efforts will continue to ensure that improvements in sensitivity and specificity of immunoassays will be widely available through commercialization.

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